Improvements in Magnetic Polymer Particles

This invention relates to magnetic polymer
particles carrying a chelating matrix loaded with a
5   metal as well as to a process for the preparation of
magnetic polymer particles carrying said chelating
matrix.   In particular, the invention relates to
magnetic polymer particles carrying a carboxymethylated
aspartate ,(Cm-Asp) chelating group and to the coupling
10   of the Cm-Asp group with the magnetic polymer particle.
Magnetic polymer particles are of general utility
in various medical and biochemical fields, for example
as transport vehicles for the delivery of pharmaceutical
products, for diagnostic purposes, for separation and
15   for synthetic purposes.   Such particles rely upon their
magnetic properties in order to perform these functions:
in diagnostic assay applications, for example,
application of a magnetic field to a sample containing
an analyte bound to magnetic polymer particles allows
20   the isolation of the analyte without the use of
centrifugation or filtration; and in therapeutic
applications, for example, application of a magnetic
field to the patient may serve to target drug-carrying
magnetic polymer particles to a desired body site.
25   By magnetic is meant herein that the polymer
particles contain superparamagnetic crystals.   Thus the
magnetic polymer particles are magnetically displaceable
but are not permanently magnetizable.   Many processes
for preparing magnetic polymer particles are known, a
30   large number of which involve preparing maghemite- or
magnetite-containing polymer particles from pre-formed
magnetic iron oxides, e.g. magnetite.   Some of processes
involved are described in US-A-4,654,267 (Ugelstad) the
contents of which are incorporated herein by reference.
35   The use of immobilised metal ion affinity
chromatography (IMAC) has been known for many years.
The IMAC purification process is based upon the

employment of a chelating matrix loaded with transition
metal ions such as Cu^{2+} or Ni^{2+} which is capable of
binding electron donating groups present on the surface
of proteins, in particular the imidazole side chain of
5   histidine. The electron donating group is believed to
coordinate to vacant coordination sites around the metal
ion. The interaction between the metal ion and the
electron donating groups present on the protein surfaces
can be altered by, for example, varying pH and hence
10   purification can be achieved via reversible metal
complex/protein interaction. Most commonly, if a
protein is bound to a solid phase via the interaction
between the metal ion and the imidazolyl side chain of
histidine, the protein can be removed by addition of
15   imidazole itself, i.e. by competitive elution.

Several different chelating ligands have been
employed in IMAC to purify proteins. Nitrilo triacetate
(NTA) (a tetradentate ligand) and the pentadentate
ligand tris(carboxymethyl)ethylenediamine are examples
20   of such ligands but these suffer from various
disadvantages such as unspecific protein interaction,
metal leakage etc.

US 6242581 proposes a solution to the metal leakage
problem by the use of a carboxymethylated aspartate (Cm-
25   Asp) group in IMAC where the bound transition metal ion
has octahedral geometry. The ligand is said to be ideal
for isolating histidine tagged recombinant proteins.
Other advantages of Cm-Asp are discussed in US 5962641,
e.g. resistance to reducing agents.

30   In these Patents the Cm-Asp ligand is bound to an
agarose solid phase which is preferably cross-linked
although other polymer matrices such as polystyrene,
nylon and SEPHAROSE are suggested. Whilst these
matrices may be magnetic the magnetic particles do not
35   remain in suspension and the solid phases are therefore
of limited use in assays.

It has now been surprisingly found that the Cm-Asp

chelating ligand can be coupled to a magnetic polymer
particle giving rise to a moiety that possesses the
ability to bind histidine-tags in recombinant proteins
or His, Cys, Met, Gln, Asn, Lys or Tyr residues present

5 in metalloprotein active sites in native proteins or
peptides, the ability to bind phosphorylated proteins or
peptides and also magnetism. This allows the skilled
biochemist more flexibility in his assaying procedures.

The inventors have also devised ways to couple the
10 Cm-Asp ligand to the magnetic polymer particles in high
yield thereby producing an excellent IMAC agent.

Viewed from a first aspect, therefore, the present
invention provides a conjugate comprising a magnetic
polymer particle bound to a carboxymethylated aspartate
15 chelating ligand.

Viewed from another aspect the invention provides a
conjugate comprising magnetic polymer particle bound to
a carboxymethylated aspartate ligand chelating a metal
atom or ion.

20 Viewed from another aspect the invention relates to
a process for the preparation of a conjugate as
hereinbefore defined comprising reacting a magnetic
polymer particle with a Cm-Asp chelating ligand.

The Cm-Asp ligand bound to the magnetic polymer
25 particle (MPP) is depicted below both in its
uncoordinated state and coordinated to a metal (the wavy
line representing a bond or a linker between the Cm-Asp
and particle):

30

4
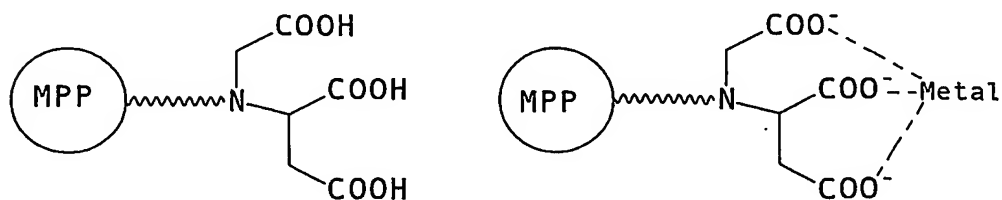
The magnetic polymer particles used in the process
of the invention may be any magnetic polymer particle
e.g. as described in US-A-4,654,267. The particles are
preferably porous to allow the presence of the
5   superparamagnetic crystals in the pores thereof. The
surface of the magnetic particles is normally
functionalised to allow coupling of the Cm-Asp ligand to
the polymer particle, e.g. it may be functionalised to
carry any known surface structure such as carboxyl
10   groups, tosyl groups, amino groups, epoxy groups,
maleamido groups, thiol groups etc. Hence, the surface
may be amine functionalized before Cm-Asp coupling.
Alternatively, an amine functionalised surface can
itself be further functionalised to carry other
15   functional groups, e.g. COOH groups.
The polymer particle is preferably made from
combinations of vinylic polymers (e.g. styrene),
acrylates and/or methacrylates. The polymeric material
may optionally be crosslinked, for example by
20   incorporation of cross-linking agents, for example as
comonomers, e.g. divinylbenzene (DVB) or ethyleneglycol
dimethacrylate. Appropriate quantities of the cross-
linking agents (e.g. comonomers) required will be well
known to the skilled man. Preferably the polymer is a
25   cross-linked styrenic polymer (e.g. a styrene-
divinylbenzene polymer, surface functionalized by the
use of a nitro-group containing comonomer, e.g. nitro-
styrene, and subsequent reduction) or a cross-linked
(meth)acrylic polymer surface functionalized by the use
30   of an epoxy-group containing comonomer (e.g.
glycidylmethacrylate) and subsequent amination (e.g. by
reaction with ethylene diamine).
The superparamagnetic crystals in the polymer
particles used in the process of the invention may be of
35   any material capable of being deposited in
superparamagnetic crystalline form in the porous polymer
particles. Magnetic iron oxides, e.g. magnetite or

maghemite are preferred; however the crystals may be of
mixed metal oxides or other magnetic material if
desired. The total quantity of crystalline magnetic
material present is generally more than 1%, preferably

5   more than 3%, desirably more than or equal to 5% (by
weight, e.g. up to 40% wt. The percentage is calculated
on a Fe (or equivalent metal in the case of magnetic
materials other than iron oxides) weight basis based
upon the overall dry weight of the coated particles.

10   Polymer particles according to the various aspects
of the present invention will generally have sizes (i.e.
diameters) that are generally in the micrometer range,
e.g. 0.3 to 100 μm, especially 0.5 to 50 μm, more
especially 0.8 to 8 μm, e.g. 0.8 to 1.2 μm.

15   Typically the porous particles used will have a
surface area of at least 15 m²/g (measured by the BET
nitrogen absorption method), and more preferably at
least 30 m²/g, e.g. up to 700 m²/g, when corrected to a
mean particle diameter of 2.7 μm (i.e. multiply surface

20 area by 2.7/MD, where MD is the mean diameter in
micrometers). Similarly scaled, the particle pore
volume is preferably at least 0.1 mL/g.

    Typically, the polymer particles are spherical and
substantially monodisperse before they are coated and

25 especially preferably remain spherical and substantially
monodisperse once they have been coated.

    By substantially monodisperse it is meant that for
a plurality of particles (e.g. at least 100, more
preferably at least 1000) the particles have a

30 coefficient of variation (CV) of less than 20%, for
example less than 15%, preferably less than 12%, more
preferably less than 11%, still more preferably less
than 10% and most preferably no more than about 8%, e.g.
2 to 5%. CV is determined in percentage as

35

$$CV = \frac{100 \times \text{standard deviation}}{\text{mean}}$$

where mean is the mean particle diameter and standard
deviation is the standard deviation in particle size.
CV is preferably calculated on the main mode, i.e. by
5   fitting a monomodal distribution curve to the detected
particle size distribution. Thus some particles below
or above mode size may be discounted in the calculation
which may for example be based on about 90% of total
particle number (of detectable particles that is). Such
10 a determination of CV is performable on a Coulter LS 130
particle size analyzer.

        Functionalisation of the polymeric material may
take place after polymerisation by, for example,
nitration and subsequent reduction of the thus-formed
15 nitro groups to pendant amine groups; or direct
amination, for example by treatment with amino ethanol.
As further alternatives, polymeric particles prepared by
the well-known Ugelstad two-step swelling process and
the improvements thereto disclosed in WO 00/61647 (Dyno)
20 may be used. Porous polymer particles produced
according to the processes described in this publication
may have magnetic particles deposited in their pores by
standard techniques.

        As a further possibility, porous polymer particles
25 may be prepared from nitro styrene and DVB, and magnetic
material introduced as taught in US-A-4,654,267.

        The superparamagnetic polymer beads sold by Dynal
Biotech ASA under the trade names Dynabeads, especially
Dynabeads MyOne are especially preferred. Dynabeads are
30 particularly advantageous since they remain in
suspension and do not exhibit magnetic particle
sedimentation often associated with other magnetic
beads. Dynabeads also show excellent magnetic mobility
compared to other magnetic particles in which high
35 levels of iron are present. Dynabeads exhibit
beneficial kinetics allowing shorter reaction times and
higher throughputs. Their unspecified binding is lower

7

than other magnetic beads and their proper
use results in a concentration of the desired material
taking place resulting in easier and more efficient
washing procedures. Finally Dynabeads, e.g. MyOne beads
5   are easy to automate and are monodisperse.

The Cm-Asp ligand is bound to the magnetic polymer
particle. By bound is meant that the ligand is
covalently linked to the polymer particle, optionally
using a linking group as discussed in detail below. The
10   Cm-Asp ligand can be bound to the magnetic polymer
particle by various procedures although it is preferred
if there are at least three linking atoms between the
polymer particle surface and the nitrogen atom of the
Cm-Asp. Preferably there are at least 5 atoms
15   separating the Cm-Asp ligand from the magnetic polymer
particle surface, more preferably there are between 6
and 20 atoms separating the Cm-Asp ligand from the
magnetic polymer particle surface.

The atoms forming the surface of the magnetic
20   polymer particle are those on the surface just prior to
coupling with the linker/Cm-Asp ligand. Thus, if the
magnetic polymer particle is activated in some way
during its manufacture (e.g. nitrated and reduced to
form an amino functionalised surface) the magnetic
25   polymer particle surface is formed by the surface
nitrogen atoms. The nitrogen atom would form the first
atom of the linker between the Cm-Asp ligand and the
particle. In a magnetic particle functionalised to
carry an electrophilic surface, e.g. a bromide surface,
30   the first atom of the linker would be that which
displaces the bromine.

Hence, in a preferred embodiment the invention
provides a conjugate of formula (I)

8

$$MPP\!\!\sim\!\!\sim\!\!\sim\!\!\sim\!\!N\!\!\begin{cases} CH_2COOH \\ CH(COOH) \\ CH_2COOH \end{cases}$$
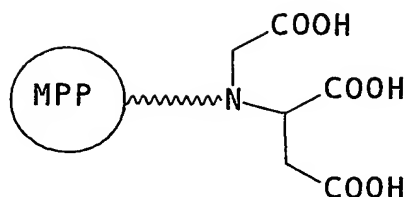
(where MPP is a magnetic polymer particle and the wavy
line represents a linking group comprising at least
three atoms, e.g. 3 to 20 atoms) or an analogue thereof
in which a metal ion is chelated.

In US 6242581 aspartic acid is coupled to the solid
phase prior to carboxymethylation to form the Cm-Asp
ligand however it has not been possible to use this
technique to provide a Cm-Asp group on a magnetic
polymer particle. Rather, the inventors have devised
alternative syntheses in which the Cm-Asp ligand is
fully formed prior to coupling to the magnetic polymer
particle.

In this regard, it has been found that when there
are fewer than 3 atoms between the polymer surface and
Cm-Asp ligand then coupling yields are low. In contrast
to an agarose support carrying Cm-Asp (as describe in
US-A-5962641), it is necessary in the present invention
to ensure that coupling yields between the magnetic
polymer particle and Cm-Asp are relatively high. The
surface area of an agarose support is considerably
greater than that of a polymer particle and hence the
binding of Cm-Asp to the (agarose) support does not need
to be achieved in high yield for a useful IMAC chelating
agent to result. In the present case, yields need to be
much higher to ensure that enough polymer particles
carry the Cm-Asp ligand and hence to ensure that IMAC
can be successfully carried out.

It is preferred if the at least 3 atom linker
comprises an amino group (-NH-). Magnetic polymer beads
are often made from styrene polymers which are nitrated
to form NO_2 groups on the surface. After reduction of

9

these groups, e.g. using ammonia, amino groups are
formed.

    The linker thus preferably comprises the residue of
an electrophile, i.e. the group which remains after

5   reaction of the electrophile with a nucleophile. Hence,
the linker may comprise an oxo group (C=O, the residue
of an ester/carboxyl group), a -CH(OH)CH$_2$- group (the
residue of an epoxide), -CH$_2$- (where the electrophile is,
for example a CH$_2$Hal). The linker may also incorporate a

10 number of atoms linking the actual electrophilic group
to the nitrogen atom of the Cm-Asp ligand, e.g. an
alkylene chain or ether chain, e.g. as in -CH$_2$CH$_2$CH$_2$-, or
-CH$_2$CH$_2$CH$_2$-O-.

    Hence the wavy line in formula (I) can represent -

15 NH-Er-N- wherein Er represents a 2 to 20 atom linker
which is an electrophile residue (Er), e.g. -(CH$_2$)$_n$-
where n is 2 to 20, which links the amino group of the
particle surface with the nitrogen atom of the Cm-Asp
ligand.

20   It is of course within the scope of the invention
for the magnetic polymer particle to carry an
electrophilic group with the Cm-Asp being functionalised
to carry a nucleophilic group. Other ways of coupling
the particle and ligand will be devised by the skilled

25 chemist.

    In an especially preferred embodiment, a particle
coating is provided which carries a carbon-carbon double
bond. This can be achieved by, for example, reaction of
the particle with an allyl or vinyl compound, e.g.

30 butenoic acid. Hydroxy functionalised particle surfaces
can be reacted with allyl bromide to form double bonds
on the particle surface. Also, carboxy functionalised
particle surfaces can be reacted with allylamines to
provide double bonds on the particle surface. The Cm-Asp

35 may then be coupled directly to the double bond using
appropriate chemistry or more preferably, the double
bond may then be reduced e.g. in the presence of aqueous

halide to provide a halide electrophile which can be reacted with the Cm-Asp ligand to ensure successful coupling.

5   Another preferred preparation process involves functionalising the surface of the magnetic polymer particle to carry carboxyl groups. The carboxylic acid groups can be activated by reaction with N-hydroxysuccinimide esters and reacted with a Cm-Asp ligand as discussed above.

10   Suitable linkers therefore include aminoalkylene, amidoalkylene, ether, ester, thioalkylene or thioester containing up to 20 atoms, e.g. NH-alkylene, NH-CO-alkylene, O-alkylene, OCO-alkylene, S-alkylene or SCO-alkylene. The nitrogen atom of the Cm-Asp ligand does
15   not form part of the linker.

The Cm-Asp ligand may too be functionalised prior to coupling with the magnetic polymer particle. For example, it has proved advantageous to provide the Cm-Asp ligand with a linking group carrying a primary
20   nucleophile to aid reaction with electrophilic groups on the particle surface. The nitrogen atom of the Cm-Asp ligand is secondary and it has been found that this atom is too unreactive, perhaps due to steric hindrance, to react in high yield with electrophilic groups, e.g.
25   halides, on the particle surface.

It is preferred therefore to couple the Cm-Asp to a linker group having at least two atoms and comprising a nucleophile such as an amine, hydroxyl or thiol group. Preferably the linker is an alkylamine, e.g. C5/6-
30   alkylamine linker or an ether/polyether linkage e.g. comprising one or two oxygen atoms and 3 to 6 carbon atoms. Coupling of the linker to the Cm-Asp (via the nitrogen atom thereof) is achieved using known chemistry as described in the Examples. The Cm-Asp ligand itself
35   can be manufactured using known chemistry. It is also possible to synthesise the entire linker CmAsp structure.

Thus, the linker CmAsp structure can be prepared
starting from a suitably protected aspartic acid
compound, e.g. where the carboxyl groups are ester
protected. This compound can be reacted with a compound
5   of formula Hal-X_1-CN (where X represents a C_{1-9} alkylene
group, and Hal a halide, e.g. Br) wherein the amino
group of the aspartic acid derivative displaces the
halide atom. The resulting secondary amino compound may
then be reacted with a Hal-CH_2COOPr type group (where Hal
10   is halide, e.g. Br and Pr a protecting group) to
introduce the final methylenecarboxy group to form the
Cm-Asp structure. Selective reduction of the nitrile,
e.g. using hydrogen and platinum (IV) oxide results in
an ideal linker which can subsequently be deprotected as
15   necessary.

Thus, viewed from a further aspect the invention
provides a process for the preparation of a compound of
formula

$$H_2N\text{---}X\text{---}N \underset{\diagdown COOR}{\overset{\diagup COOR}{\overset{\diagup COOR}{\mid}}}$$
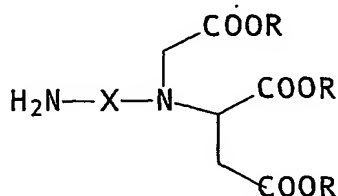
20

(wherein each R independently represents hydrogen or a
protecting group and X represents an C_{2-20} alkylene
linker, especially a $C_{5/6}$-alkylene);

comprising reacting a compound of formula Hal-X_1-CN
25   (wherein Hal is a halide and X_1 represents an C_{1-19}
alkylene linker) with a compound of formula

$$H_2N \underset{\diagdown COOPr}{\overset{\diagup COOPr}{\mid}}$$
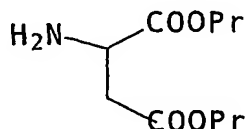
(wherein Pr represents a protecting group)
30   reacting the resulting product with a compound of
formula Hal-CH_2COOPr to form a compound

$$\text{NC}-\text{X}_1-\text{N}\underset{\diagdown \text{COOPr}}{\overset{\diagup \text{COOPr}}{\diagup \text{COOPr}}}$$
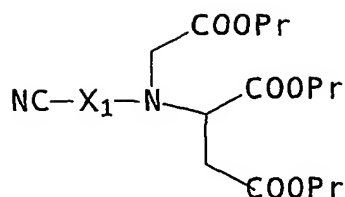
5   and reducing the nitrile to an amino group, preferably
without removing the protecting groups. These can then
be removed as necessary. The skilled chemist will
realise that the X linker has one more carbon atom than
the X. linker which derives from the nitrile.

    skilled chemist will be able to devise further
    for synthesising the Cm-Asp linker molecules of
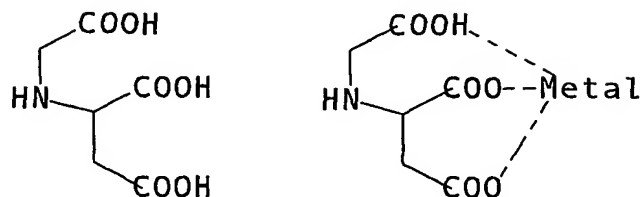   he invention.
    wed from another aspect the invention provides a
    for the preparation of a conjugate comprising a
     polymer particle bound to a Cm-Asp ligand
    ng reacting a Cm-Asp ligand of formula (II)
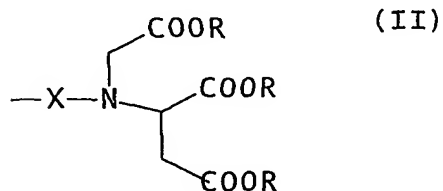
$$-\text{X}-\text{N}\underset{\diagdown \text{COOR}}{\overset{\diagup \text{COOR}}{\diagup \text{COOR}}} \qquad \text{(II)}$$

(wherein each R independently represents hydrogen or a
protecting group and X represents a 2 to 20 atom linker,
e.g. an C_{2-10} alkylene linker, especially a $C_{5/6}$-alkylene
linker) with a magnetic polymer particle, e.g. one
25   functionalised to carry an electrophilic coating, e.g.
an ester, epoxide, allyl, alkyl halide etc coating.
       Compounds of formula (II) and analogues thereof in
which a metal is chelated are themselves new and form a
further aspect of the invention along with the Cm-Asp
30   ligand itself, i.e. a compound of formula (III) and its

analogue where a metal is chelated.

$$\begin{array}{cc}
\text{HN} < \begin{array}{l} \text{COOH} \\ \text{COOH} \\ \text{COOH} \end{array} & \text{HN} < \begin{array}{l} \text{COOH} \cdots \\ \text{COO--Metal} \\ \text{COO} \end{array}
\end{array}$$

In some embodiments of the invention it may be necessary to protect the carboxyl groups of the Cm-Asp ligand during syntheses. This can be easily effected using known protection strategies, e.g. using an ester protecting group which can be hydrolysed in acid or base as is known in the art.

The Cm-Asp ligand can coordinate any metal atom or ion. By metal is meant any metal from groups 1 to 13 of the periodic table, a lanthanide or actinide or a metal Si, Ge, Sn, Pb, As, Sb, Bi, Te, Po or At. The metal should preferably be an ion and preferably be a transition metal or a metal of group 13. Preferred metal ions are those in the 2+ or 3+ oxidation states. Where the metal ion is in the 2+ oxidation state, the entire particle-linker-ligand-metal ion assembly is uncharged which reduces the possibility of non-specific binding.

Preferred metals are Ni, Fe, Ga, Mn, Co, Cu and Zn of which Fe, Ga, Mn and Co are preferred, especially Co^{2+}. Coordination can be easily effected by exposing the Cm-Asp to, for example, a metal chloride.

The conjugates with associated metal can in general be used for attaching to and combining with peptides, proteins or other polymers (e.g. antibodies) and are hence of use in a wide variety of assays. They are of particular use, however, in the isolation of proteins/peptides tagged or native by immobilised metal ion affinity chromatography. In particular they are of use in the isolation of histidine-tagged recombinant

14

proteins/peptides, His, Cys, Met, Gln, Asn, Lys and/or
Tyr containing native proteins and/or peptides, and
phosphorylated proteins or peptides. Especially
preferably, the conjugatges are of use in the isolation
5   of histidine-tagged recombinant proteins/peptides.
Hence viewed from another aspect the invention provides
the use of a conjugate comprising a magnetic polymer
particle bound to a Cm-Asp ligand, said ligand
coordinating a metal atom or ion, in an assay. . Suitable
10 assays and ways to carry these out are known by the
skilled biochemist.

For example, the capture of histidine-tagged
on the Cm-Asp functionalised particles of the
n has various applications. The rapid reaction
and gentle handling of isolated proteins make
hnology well suited for the "pull down" of large
complexes. Thus Cm-Asp functionalised beads may
in sample preparation for mass spectrometry
. It is believed that complexes isolated with
sp functionalised beads may be more intact than
s isolated with columns or other solid supports
g other magnetic particles with uneven surfaces
therefore ideal for use in mass spectrometry
solation.
Cm-Asp technology may also act as a solid phase
for use in assay procedures. The Cm-Asp beads are not
prone to aggregation and are highly dispersed in
solution and show a low degree of non-specific binding.
These properties allow for high quality screening
30 results and protocols that are easily automated on a
wide range of automation platforms. The Cm-Asp beads
may also be used in phage display perhaps as a solid
phase or to purify expressed phage display selected
proteins from a library.
35   In general therefore the capture of histidine
tagged proteins and/or His, Cys, Met, Gln, Asn, Lys
and/or Tyr residue containing native proteins or

peptides may allow microscale protein purification, clean up of mutated protein libraries, denaturing elution of protein/peptide, mild elution of proteins/peptide, protein-protein interaction studies

5   and screening technologies, e.g. for drug discovery, molecular display, aptamer screening, phage display, engineered enzyme screening and diagnostics.
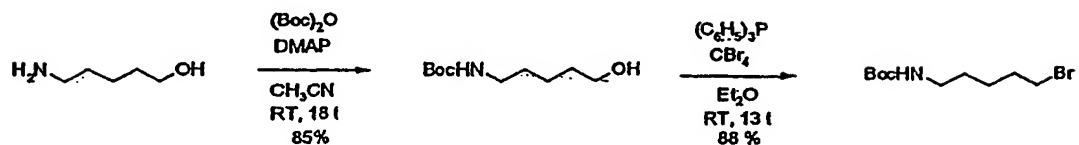
The invention will now be described further by reference to the following non-limiting examples.

10

Example 1
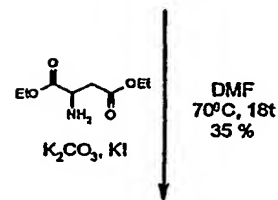
<u>Synthesis of the Cm-Asp ligand</u>
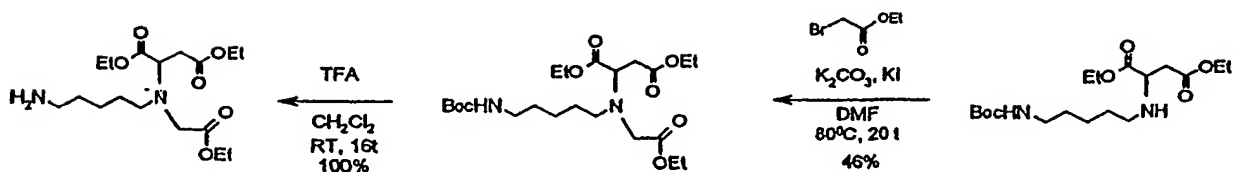
5　　The Cm-Asp triester below is prepared as follows:
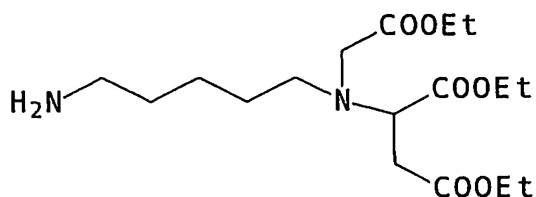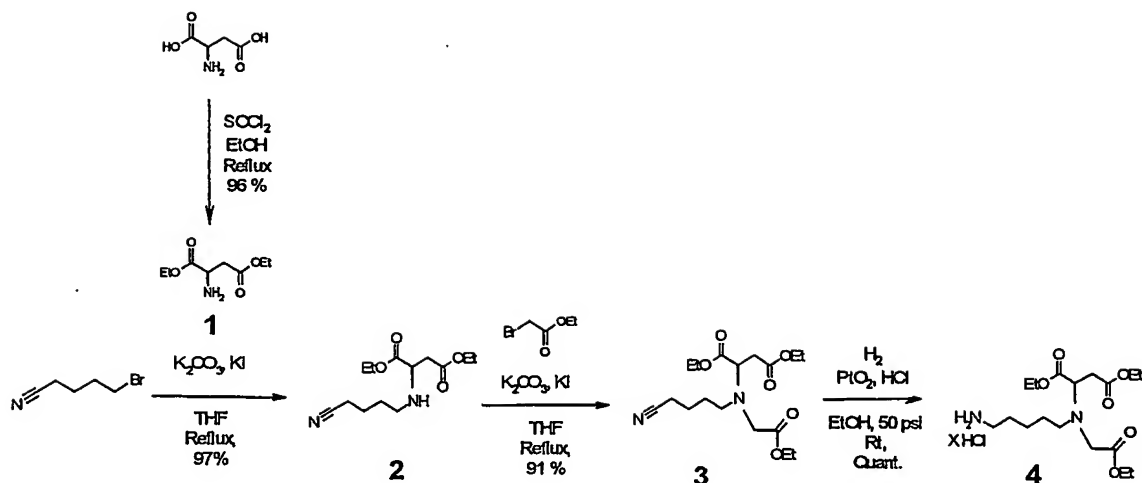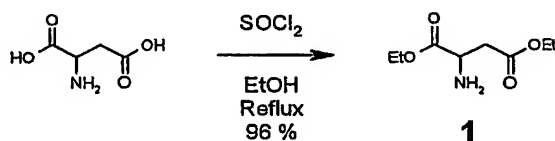

10

15

20

25

Example 2

Alternative Synthesis of Cm-Asp triester

30

5   **Synthesis of 2-amino-succinic acid diethyl ester**

10   To a suspension of DL-aspartic acid (91.5 g, 0.69 mol)
     in abs. ethanol (800 ml) at 0°C thionylchloride (150 ml,
     2.06 mol) was added dropwise. The cooling bath was
     removed and the mixture refluxed for 3 hours. After
     cooling to ambient temperature the solvent was
15   evaporated *in vacuo* and to the residue added a saturated
     aqueous solution of K_2CO_3 to pH 8. The aqueous phase was
     extracted with ethyl acetate (x 3) and the combined
     organic phases washed with brine and dried ($MgSO_4$), prior
     to filtration and evaporation *in vacuo* to give 124.8 g
20   (96 %) of compound **1** as an yellow oil. The crude product
     was used directly in the next step.

     ^{1}H NMR (200 MHz, $CDCl_3$): 4.06 (m, 4H), 3.68 (m, 1H), 2.61
     (m, 2H), 1.73 (s, 2H), 1.06 (m, 6H).

### Synthesis of 2-(4-cyano-butylamino)-succinic acid diethyl ester

2
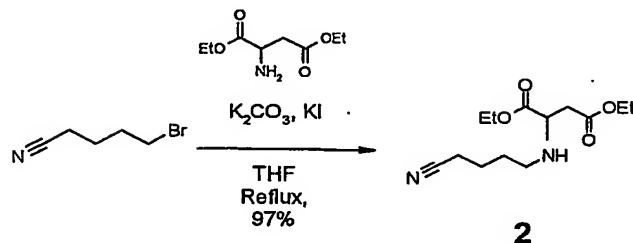
To a suspension of **1** (93.0 g, 0.49 mol), K_2CO_3 (34.0 g, 0.25 mol), and KI (12.3 g, 0.07 mol) in THF (600 ml) 5-bromovaleronitrile (28.4 ml, 0.25 mol) was added dropwise. The reaction mixture was heated to reflux and stirred for 5 days. After cooling to ambient temperature the mixture was filtered, and the filtrate evaporated *in vacuo*. Purification on silica gel, eluting with hexane/ethyl acetate (7:3) afforded 64.1 g (97 %) of compound **2** as an yellow oil.

^{1}H NMR (200 MHz, CDCl$_3$): 4.06 (m, 4H), 3.40 (t, 1H), 2.50 (m, 4H), 2.25 (t, 2H), 1.45 (m, 4H), 1.15 (m, 6H).

Synthesis of 2-[(4-cyano-butyl)-ethoxycarbonylmethyl-amino]-succinic acid diethyl ester
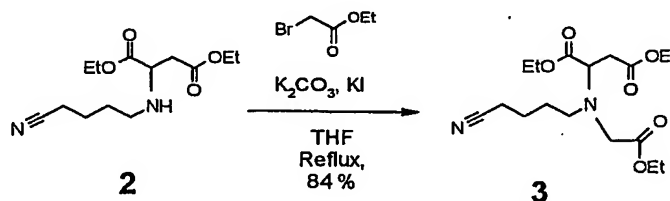
To a mixture of **2** (86.6 g, 0.32 mol), K_2CO_3 (44.3 g, 0.32 mol), and KI (16.0 g, 0.10 mol) in THF (650 ml) ethyl bromoacetate (42.5 ml, 0.38 mol) was added. The reaction mixture was heated to reflux and stirred for 5 days.

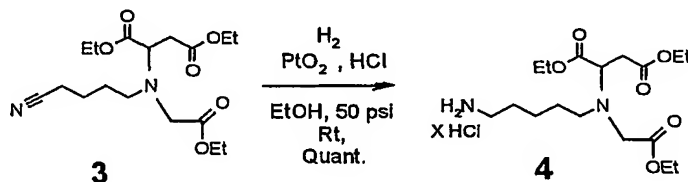After cooling to ambient temperature the mixture was filtered, and the filtrate evaporated *in vacuo*. Purification on silica gel, eluting with hexane/ethyl acetate (8:2) afforded 103.7 g (91 %) of compound **3**.

^{1}H NMR (200 MHz, CDCl$_3$): 4.18 (m, 6H), 3.91 (t, 1H), 3.42 (s, 2H), 2.77 (m, 4H), 2.40 (t, 2H), 1.65 (m, 4H), 1.25 (m, 9H).

Synthesis of 2-[(5-amino-pentyl)-ethoxycarbonylmethyl-amino]-succinic acid diethyl ester

To a solution of **3** (15 g, 42 mmol) in 95% ethanol (60 ml) and concentrated HCl (10 ml) a suspension of PtO$_2$ (600 mg, 2.6 mmol) in 95% ethanol (20 ml) was added. The reaction mixture was hydrogenated at 50 psi overnight. The mixture was filtrated and the filtrate evaporated *in vacuo* and pumped overnight to afford a quantitative yield of the title compound as the HCl-salt.

^{1}H NMR (200 MHz, D$_2$O): 4.82 (t, 1H), 4.20 (m, 6H), 3.53 (q, 4H), 3.34 (m, 2H), 3.18 (b d, 2H), 2.92 (b t, 2H), 1.65 (m, 4H), 1.10 (b m, 9H).

Example 3:

Bromination

17.3 g of a methanol suspension of the magnetic styrene particles having 0.5 mmol/g allyl groups were washed four times with 45 mL sodium acetate buffer (pH = 5.9). After adjusting the particle content to 9 wt%, 0.96 g of
5   pyridinium tribromide dissolved in 10 mL DMF was added while stirring at 350 rpm. After five minutes at room temperature the particles were washed five times with 45 mL deionised water.

10   **Example 4:**

Functionalization with Cm-Asp chelator

18.0 g of a suspension of the particles prepared as in Example 3 were washed three times with 20 mL of 50mM
15   sodium bicarbonate. The particle content was adjusted to 12 wt%. To the suspension 0.17 g of the Cm-Asp triester (prepared as described in Example 1) was added. 50mM sodium bicarbonate was added until a particle content of 10 wt% was achieved. The reaction mixture was shaken at
20   600 rpm at 40°C for 15 hours. The particles were then washed four times with 20 mL deionised water.

     Example 5:

Hydrolysis

25

20.0 g of a suspension of particles prepared as in Example 4 were washed twice with 20 mL of 1M lithium hydroxide. After adjusting the particle content to 10 wt% the mixture was shaken at 250 rpm for four hours at
30   room temperature. The particles were then washed with deionised water until pH 6-7.

     Example 6:

General Metal-loading conditions

35

250 mg of particles prepared as in Example 5 are washed

twice with 5 ml reverse osmosis-water followed by 15 min
sonication. 5 ml of 10 mM metal salt (MX) are added to
the particles and incubated for 30 min. The tube is
placed in a magnet, and the supernatant is removed. The
particles are washed twice with 5 ml phosphate buffered
saline (0,01% Tween 20, pH 7,4). The particles are then
washed once in 20% ethanol. The particles are stored in
20% ethanol.

The following metal salts were employed MX = $CoCl_2$,
$CuSO_4$, $FeCl_3$, $GaCl_2$, $GaCl_3$, $MnSO_4$, $MgCl_2$, $NiCl_2$, $CaSO_4$,
$ZnCl_2$

Example 7

Functionalisation of carboxylic acid groups to N-
hydroxysuccinimide ester

50 g of a suspension of 5.0 g of the particles of MyOne
Carboxylic acid beads (Dynal Biotech ASA) were acidified
by washing with 0.1 M acetic acid (3 x 50 mL). The
acidified particles (which have a carboxylic acid
content of 0.5 mmole/g DS) were then washed with acetone
(4 x 50 mL) and concentrated on a magnet. Extra acetone
was added until a total of 35.6 g suspension is
achieved. N-hydroxysuccinimide (2.90 g, 25 mmole) and
diisopropylcarbodiimide (3.16 g, 25 mmole) were then
added. The reaction mixture was stirred at room
temperature for 5 hours. The particles were then washed
with acetone (5 x 50 mL).

Example 8:

Functionalization with Cm-Asp chelator

44 g of an acetone suspension of the beads of Example 7,
were washed three times with 50 mL isopropanol. After
adjusting the particle content to 12 wt%, 5.6 g of

triethylamine was added. 0.10 g of the Cm-Asp triester
(prepared as described in Example 1) dissolved in
isopropanol, was then added. This results in a particle
content of 10 wt%. The reaction mixture was then shaken

5   at 250 rpm at room temperature for 20 hours. The
particles were washed three times with 50 mL of
isopropanol.

Example 9:

10   Functionalization with Cm-Asp chelator and ethanolamine

To 10 g of an isopropanol suspension of the particles
prepared as in Example 8, 0.32 g of ethanolamine was
added. The reaction mixture was then shaken at 250 rpm

15   at room temperature for 18 hours. The particles were
then washed three times with 10 mL of isopropanol.

Example 10

Functionalization with Cm-Asp chelator

20

1,2 gram of dry Dynabeads 270 Epoxy were mixed with 8,8
gram of 50 mM sodium bicarbonate. To the suspension 0,17
grams of the Cm-Asp triester (prepared as described in
Example 1) were added, and the reaction mixture was .

25   shaken at 600 rpm at 60°C for 16 hours. The particles are
worked up by washing four times with 20 ml deionised
water.

Example 11

30   Purification of Histidine-tagged recombinant proteins

1.   2 mg of a suspension of particles with Co^{2+} prepared
as in Example 6 were washed with 700 μl 50 mM Na-
phosphate, pH 8.0, 300 mM NaCl, 0.01% Tween®-20.

35   2.   The supernatant was removed and the particles were
resuspended in 100 μl of the same buffer as in step 1.

3.   A suspension of lysed E. coli cells with expressed recombinant histidine-tagged protein was added to the particle suspension. The total volume was adjusted to 700 μl with the same buffer as in step 1. This

5   suspension was incubated for 10 minutes.

4.   The supernatant was removed and the particles with the bound histidine-tagged protein were washed four times with 700 μl of the same buffer as in step 1.

5.   The histidine tagged protein was eluted in 100 μl

10   150 mM Imidazole, 50 mM Na-phosphate, pH 8.0, 300 mM NaCl, 0,01% Tween®-20.

6.   The purified protein was analysed by SDS-Tris-HCl polyacrylamide gel and bromphenol blue staining.

15

Example 12

<u>Purification of phosphorylated peptides</u>

1.   2 mg of a suspension of particles with Fe^{3+} prepared

20   as in Example 6 were washed twice with 500 ml 5% acetic acid.

2.   The supernatant was removed and 100 μl 10% acetic acid was added. 100 μl of 30 μg trypsinated b-casein was added. This was incubated for 30 min.

25   3.   The supernatant was removed and the particles with the bound phosphorylated peptides were washed twice with 250 μl 1% acetic acid.

4.   The supernatant was removed and the particles with the bound phosphorylated peptides were washed twice with

30   250 μl 0,1% acetic acid, 10% acetonitrile.

5.   The supernatant was removed and the particles with the bound phosphorylated peptides were washed with 250 μl H_2O.

6.   The phosphorylated peptides were eluted in 50 μl

35   0,1 M ammonium bicarbonate.

7.   The purified phosphorylated peptides were analysed by HPLC.

Example 13

<u>Purification of metal binding proteins</u>

5

1. · 2 mg of a suspension of particles with Mn^{2+} prepared as in Example 6 were washed twice with 250 µl Acetate buffer pH 4.0, 250 mM NaCl.

2.   The supernatant was removed and 250 ml of the same

10   buffer as in step 1 was added. 30 µg (3 µl) of an Mn binding protein was added. This was incubated for 10 min.

3.   The supernatant was removed and the particles with the bound protein were washed twice with 250 µl of the

15   same buffer as in step 1.

4.   The Mn-binding protein was eluted in 50 µl 0,1 M ammonium bicarbonate.

5.   The eluted protein was analysed by SDS-Tris-HCl polyacrylamide gel and silver staining.

20